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# Evaluation of STP2-dependent $\alpha$ -Synuclein Toxicity in Yeast: Role of GAPDH?

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## Summary

Parkinson disease (PD) is caused by  $\alpha$ -synuclein misfolding in the substantia nigra par compacta cells of the brain. A recent study (Willingham et al., 2003) demonstrated that over expression of wild type (WT)  $\alpha$ -synuclein in STP2 deletion (STP2 $\Delta$ ) *S. cerevisiae* strains is toxic. Surprisingly, we did not observe any toxicity in 4741 (parent strain) and STP2 $\Delta$  strains over expressing WT  $\alpha$ -synuclein or its mutant forms. Moreover, GFP microscopy of  $\alpha$ -synuclein in STP2 $\Delta$  strains showed phenotypes observed in strains with STP2. We observed halos for strains over expressing WT  $\alpha$ -synuclein and the A53T mutant.  $\alpha$ -synuclein was cytoplasmic for strains with the A30P mutant and the A30P/A53T double mutant. Western analysis demonstrated that neither the levels of  $\alpha$ -synuclein expression nor the levels of GAPDH, a pro-apoptotic protein changed in STP2 $\Delta$  strains. Furthermore, genetic deletion of GAPDH did not show any enhanced growth in strains that overexpressed  $\alpha$ -synuclein. The lack of  $\alpha$ -synuclein toxicity in STP2 $\Delta$  in our study may be due to our use of a different overexpression system, which did not yield amounts high enough to overwhelm protein quality-control systems and yield the toxicity that underlies PD pathogenesis (Berke, 2003) of the cell.

## Introduction

Described first by James Parkinson (1817), PD is one of the most common neurodegenerative diseases in the world and affects about 4 million people worldwide (Dauer, 2003). Clinically, the major symptoms are tremor at rest, slowness of movement (bradykinesia), reduced facial expressions, and balance problems (Lang et al., 1998). Pathologically, PD is caused by the neurodegeneration of the substantia nigra compacta (SNc), resulting in the loss of dopaminergic neurons (Colcher A., 1999). The major clinical hallmark is the presence of eosinophilic inclusions known as Lewy Bodies (LBs) that forms an array of radiating fibrillar structures (Spillantini et al., 1998). Ultra structural analysis had revealed that LBs are amyloid like fibrils consisting of misfolded  $\alpha$ -synuclein proteins (Spillantini et al., 1998). One can acquire PD by either inheriting the particular genetic mutation or sporadically. In familial forms, the misfolding of  $\alpha$ -synuclein is associated with the substitution of the 53<sup>rd</sup> Threonine (Polymeropoulos et al., 1997) and the 30<sup>th</sup> Proline (Kruger, 1998) by an alanine.

The normal function of  $\alpha$ -synuclein is still not well known. But evidences do support their role in membrane plasticity (Clayton et al., 1998). The stabilization of its secondary structure upon binding to synthetic membranes (Davidson et al., 1998), the interaction of  $\alpha$ -synuclein and phospholipids (Perrin et al., 2000), and the membrane

binding characteristics of  $\alpha$ -synuclein in cultured cells (Cole et al., 2002) suggests its role in the plasma membrane. Most recent studies have discovered the lipid-binding domain of  $\alpha$ -synuclein and propose its roles in the regulation of dopamine transporter activity (Wersinger et al., 2003).

In 2003, Willingham et. al investigated the different upstream and downstream factors linked with  $\alpha$ -synuclein. They showed 86 yeast genes that enhanced  $\alpha$ -synuclein and huntingtin toxicity in their absence. Among them, one was STP2. However, the study did not shed further light on the connections between  $\alpha$ -synuclein, huntingtin and STP2. Structurally, STP2 or N-hydroxyarylamine sulfotransferase 2 belongs to the family of phenol preferring sulfotransferases (Khan et al., 1995). Nevertheless, recent studies have reported its role along with STP1 (Andersson, 2002) as a transcription factor for amino acid permease genes (de Boer et al., 2000) that transport extra-cellular amino acids to the cell (Nielsen et al., 2001). Most recent study by Eckert-Boulet et. al., (2004) further expanded the role of STP2. Transcriptional profiling of STP2/STP1 knockout strains of *S. cerevisiae* using DNA micro array showed the up regulation of several carbohydrate metabolic proteins such as glyceraldehydes-3-phosphodehydrogenase (GAPDH3 or Hsp35) and glucokinase (Eckert-Boulet et al., 2004).

GAPDH3 is one among the three isoforms of GAPDH protein, which is an enzyme that converts glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate in the step 6 of glycolysis (McAlister, 1985). Recent studies have revealed the diverse roles of GAPDH in the neurodegeneration of dopaminergic neurons (Fukuhara et al., 2001; Mazzola, 2002). Treating neurodegenerative cells with known GAPDH inhibitors such as CGP 3466B (Waldmeier, 2000), rasagiline (Maruyama, 2001), and deprenyl & propargylamines (Tatton, 2003) have shown rescuing effects. These evidences suggest that GAPDH may play a role in the signal transduction pathways that control neuronal apoptosis (Tatton (2), 2003). Among the three isoforms of GAPDH, studies have also shown the link between GAPDH3 or Hsp35 and protein misfolding during stress (Delgado, 2003). A specific connection between  $\alpha$ -synuclein and GAPDH was shown by the demonstration of the localization of GAPDH with the LBs of PD affected patients (Tatton et al., 2000).

Our study aims to investigate the connection between  $\alpha$ -synuclein and STP2. Based on the link between STP2 and GAPDH (Eckert-Boulet et al., 2004), we hypothesize that the enhancement of  $\alpha$ -synuclein toxicity in STP2 $\Delta$  strains, as demonstrated by Willingham et al., (2003) was due to elevated GAPDH levels. We examined the toxicity of parent and STP2 $\Delta$  strains over expressing WT  $\alpha$ -synuclein. Furthermore, we extended the study by over expressing the mutant forms of  $\alpha$ -synuclein too. To investigate the role of GAPDH in the toxicity observed by Willingham et al., (2003), we examined the expression levels of GAPDH in STP2 $\Delta$  strains. Lastly, to investigate the possibility of enhanced growth by GAPDH inhibition, we examined GAPDH over expressing WT  $\alpha$ -synuclein and its mutant forms.

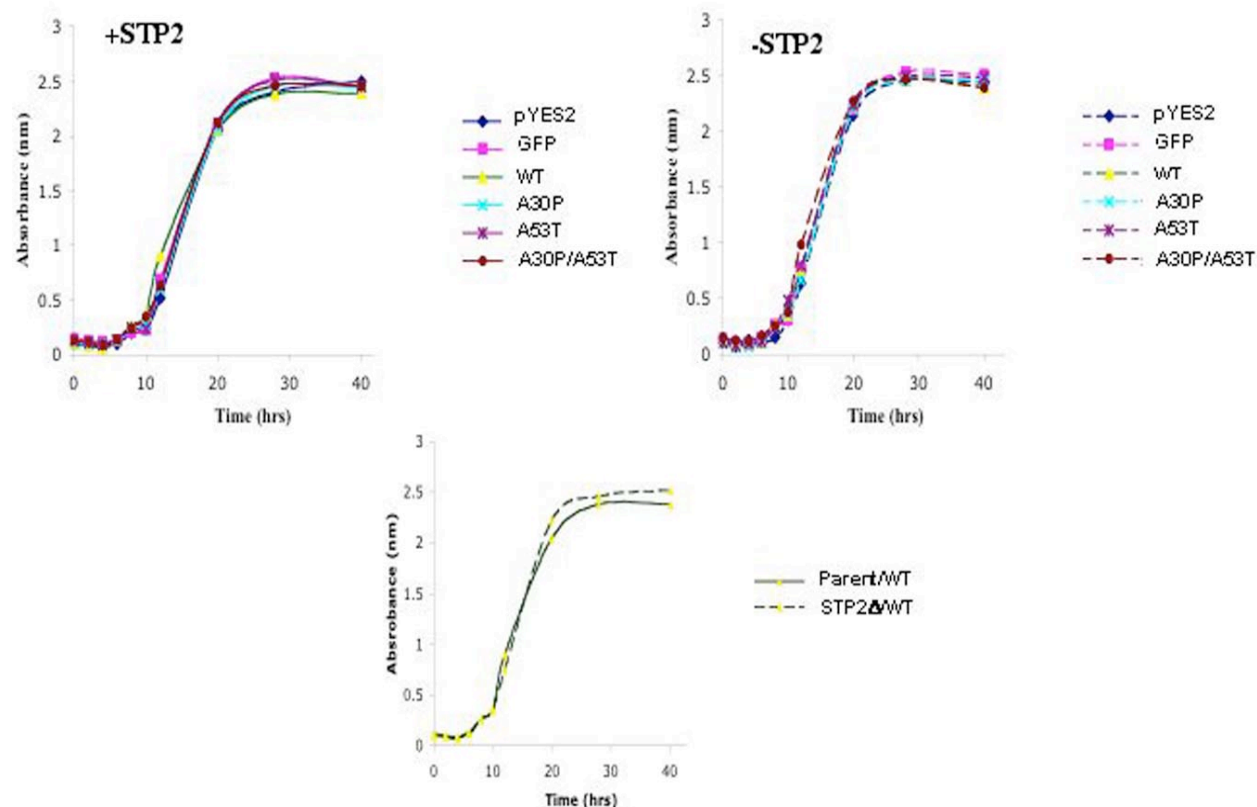
## Results

### $\alpha$ -Synuclein toxicity is not enhanced by STP2 Deletion

\* This paper was written based on original scholarship conducted in BIO324 Advanced Cell Biology, by Dr. Shubik DebBurman.

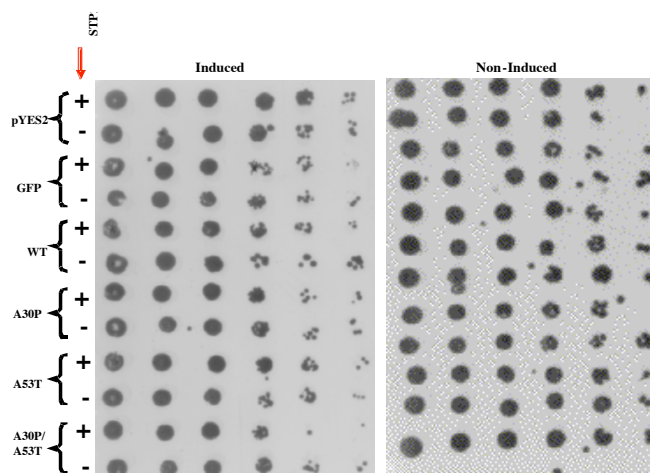
We first asked if WT  $\alpha$ -synuclein toxicity was enhanced in the absence of STP2. Surprisingly, the growth curves of parent and STP2 $\Delta$  strains over expressing WT  $\alpha$ -synuclein do not demonstrate any toxicity (Figure 1). For further confirmation, we conducted a yeast viability assay (serial spotting) and still did not observe any toxicity as shown in Fig. 2. We then repeated the experiments on parent and

STP2 $\Delta$  strains over expressing the familial mutants of  $\alpha$ -synuclein-A30P & A53T, and an artificial double mutant A30P/A53T. However, both assays (Fig. 1 & Fig. 2) demonstrated the absence of any toxicity in any of the STP2 $\Delta$  strains. Our controls were strains with the parent plasmid (pYES2) and GFP alone.



**Figure 1. Growth Curves of parent and STP2D Strains**

Yeast cells were grown in SC-URA/Galactose for  $\alpha$ -Synuclein expression. Absorbance of the growing culture at 0hrs, 3hrs, 6hrs, 9hrs, 12hrs, 18hrs, 24hrs, and 36hrs were taken using the Hitachi U-200 spectrophotometer at 600nm. The growth curves of 4741(left, lines) and STP2D (right, dashed lines) strains shows the absence of any toxicity. Both graphs were plotted to the same scale as demonstrated by the graph in the bottom.



**Figure 2. Serial Spotting of Parent and STP2D Strains**

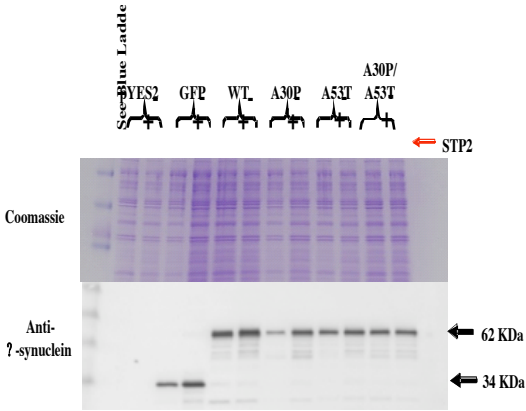
Yeast cells were grown to mid-log phase in SC-URA raffinose. They were then normalized to equal densities ( $2 \times 10^7$  cells/ml), serially diluted (five fold), and spotted on SC-URA glucose (left) and galactose (right) plates. The assay indicates the absence of toxicity in both parent and STP2Δ strains over expressing WT  $\alpha$ -synuclein, A30P, A53T, and A30P/A53T. pYES2 and GFP alone were used as controls.

**$\alpha$ -Synuclein expression and size unchanged in STP2Δ strains**

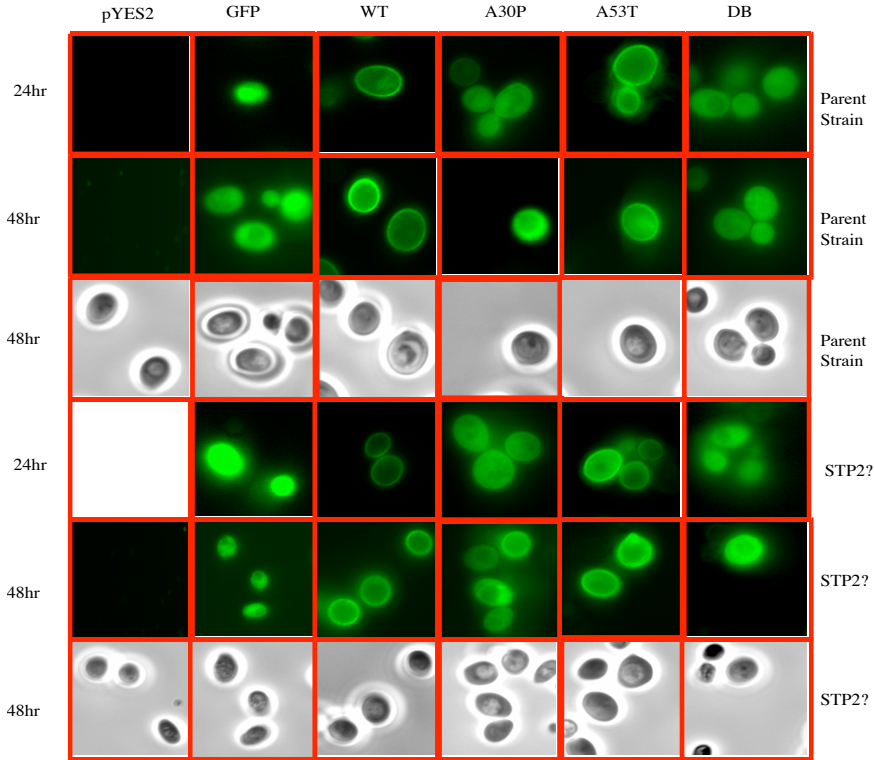
To investigate the possibilities of  $\alpha$ -synuclein aggregation and differences in  $\alpha$ -synuclein expression, we examined the lysates of parent and STP2Δ strains by Western analysis. The immunoblotting shows no signal for the parent vector pYES2 and the expected band of size 34 kDa for GFP alone (Sharma, 2004). Equal amount of  $\alpha$ -synuclein expression was observed for the parent and STP2Δ strains over expressing WT  $\alpha$ -synuclein and its mutant forms (Figure 3). The minor differences between the different strains could be accounted by the differences in the protein content as demonstrated by coomassie staining (Figure 3). The immunoblotting also did not show  $\alpha$ -synuclein aggregates (Figure 3), since the bands were of expected size with ~62kDa (Sharma, 2004). We also did not see any differences in either expression level or in the size of  $\alpha$ -synuclein in strains over expressing the mutant forms of  $\alpha$ -synuclein (Figure 3).

**Cytoplasmic and peripheral localization of  $\alpha$ -synuclein unvaried in STP2Δ**

GFP microscopy was conducted at the 24<sup>th</sup> hour after induction on all the parent and STP2Δ strains to examine the phenotypes of  $\alpha$ -synuclein expression (Rows 1 & 2; Figure 4). Strains with the parent plasmid and GFP alone were used as controls (Columns 1 & 2; Figure 4). To evaluate phenotypic changes with time, we examined the strains at the 48<sup>th</sup> hour also ( Rows 2, 3, 5 & 6; Figure 4 ). Extensive



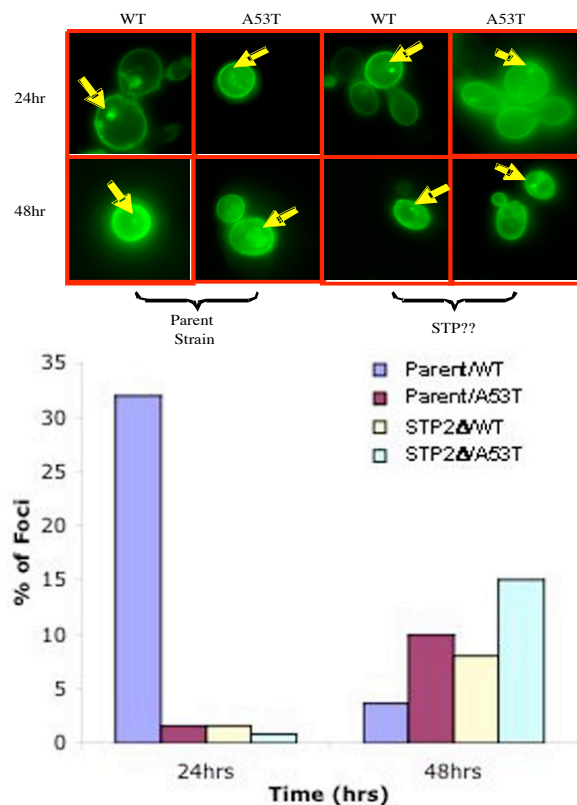
**Figure 3. Western Analysis of Parent and STP2Δ Lysates**  
Yeast cells ( $2.5 \times 10^7$  cells/ml) were washed in 50mM Tris (pH 7.5), 10mM NaCl and solubilized in Electrophoresis Sample Buffer (ESB; Burke, 2000). Samples (25  $\mu$ l) were run on pre-cast 10-20% Tris-Glycine SDS gels (Invitrogen), using See Blue (15  $\mu$ l) as the molecular standard (Invitrogen). Gels were transferred to PVDF membranes (BioExpress) and probed with the antibodies: anti- $\alpha$ -synuclein (Santa Cruz, Biotech). To detect the antibodies, the blot was treated with color development solution. WT  $\alpha$ -synuclein, A30P, A53T, and A30P/A53T migrated to 62 KDa and GFP alone until 34 KDa. The blot shows the absence of difference in neither size or expression levels of  $\alpha$ -synuclein.



**Figure 4. GFP Analysis of Parent and STP2Δ Strains**

To analyze cells under fluorescent microscopy, expression was induced for 24 hours and was diluted to a density of  $5 \times 10^6$  cells/ml in 25ml of SC-URA/Galactose media. Cells were grown to log-phase, and resuspended in 250ul of SC-URA/Glucose media to concentrate them 100-fold. The figure shows intense cytoplasmic localization for all the strains with A30P mutant and the double mutant A30P/A53T and bright halos for all the strains with WT  $\alpha$ -synuclein and the A53T mutant. No phenotypic difference was observed at 48hrs after induction.

cytoplasmic localization of  $\alpha$ -synuclein was observed in the parent and STP2 $\Delta$  strains over expressing GFP alone, WT  $\alpha$ -synuclein and the A30P mutant (Columns 2, 3, and 4; Figure 4). However, halos could be seen for strains over expressing A53T and A30P/A53T suggesting the localization of  $\alpha$ -synuclein in the plasma membrane (Columns 5 and 6; Figure 4). Among the different phenotypes we investigated, intense cytoplasmic localization of  $\alpha$ -synuclein in the form of spherical bodies also known as foci was seen in the strains over expressing the WT  $\alpha$ -synuclein and the A53T mutant (Figure 5; top). Quantification of cells with foci at the 24<sup>th</sup> and 48<sup>th</sup> hours shows that the % of foci decreases for the parent strain over expressing WT  $\alpha$ -synuclein and increased for the A53T mutant (Figure 5; bottom). An increase in the % of foci was also seen in the STP2 $\Delta$  strains overexpressing WT  $\alpha$ -synuclein and the A53T mutant (Figure 5; bottom).



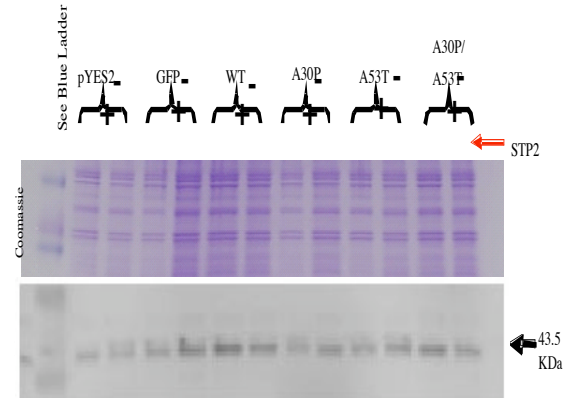
**Figure 5. GFP Analysis Reveals Foci**

The cells were prepared as in Fig. 4. Foci were observed in in all the strains overexpressing WT and A53T  $\alpha$ -synuclein. Quantification was carried out by counting cells in the number of foci in six adjacent fields. The number of foci decreased for parent strain with WT  $\alpha$ -synuclein at the 48th hour, but increased for the STP2 $\Delta$  strains with WT  $\alpha$ -synuclein and A53T mutant (bottom). An increase in the % of foci was also seen in the parent strain with the A53T mutant (bottom).

#### GAPDH Levels Remains Unchanged in STP2 $\Delta$ strains

We hypothesized that the enhanced toxicity observed by Willingham et. al., (2003) was due to elevated levels of GAPDH. To test this hypothesis, we investigated the expression levels of GAPDH by Western analysis on the cell lysates of parent and STP2 $\Delta$  strains over expressing WT  $\alpha$ -

synuclein (Figure 6). The immunoblotting shows the GAPDH band with expected size of 43.5Kda. However, we observed the absence of any difference in GAPDH expression between the parent and STP2 $\Delta$  strains. We also did not see any difference between the parent and STP2 $\Delta$  strains over expressing the mutant forms of  $\alpha$ -synuclein--A30P & A53T and the A30P/A53T. The cell lysates of strain with the parent plasmid and GFP alone were used as controls.

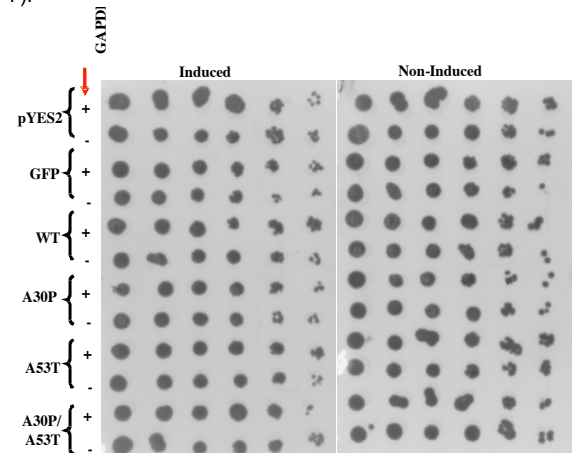


**Figure 6. Western Analysis of Parent and STP2 $\Delta$  Lysates Examining GAPDH Expression**

The Western was conducted as described in Figure 3. The blot was probed with the primary anti- GAPDH antibody (ChemiCon International) followed by the secondary anti-PGK (Molecular Probes) antibody. As expected, the GAPDH protein migrated till 43.5 KDa. The blot shows the absence of difference in GAPDH expression between Parent and STP2 $\Delta$  strains.

#### Deletion of GAPDH does not enhance growth

Although we did not observe any toxicity in STP2 $\Delta$  strains, we asked whether the deletion of GAPDH might enhance growth in a STP2 independent manner. To investigate this possibility, we used a strain lacking GAPDH3. We compared the growth of parent and GAPDH $\Delta$  strains over expressing WT  $\alpha$ -synuclein and it mutant forms by serial spotting (Figure 7). Further extending the study with GAPDH $\Delta$  over expressing A30P, A53T, and A30P/A53T also did not shown any enhancement in growth. The strains with the parent plasmid and GFP alone were again used as controls (Figure 7).



**Figure 7. Serial Spotting of Parent and GAPDH $\Delta$  Strains**

The spotting was conducted as described in Figure. 2. The assay indicates the absence of any toxicity in both parent and GAPDH $\Delta$

strainover expressing WT  $\alpha$ -synuclein, A30P, A53T, and A30P/A53T. pYES 2and GFP alone were used as controls.

## Discussion

In this study, we investigated the enhanced  $\alpha$ -synuclein toxicity observed by Willingham *et. al.*, (2003) in STP2 deletion strains of *S. cerevisiae* and extended this analysis to familial mutants. We proposed this enhanced toxicity was due to increased GAPDH levels. However, we neither observed toxicity nor difference in GAPDH levels in STP2 $\Delta$  strains.

### STP2 toxicity may require higher expression of $\alpha$ -synuclein

Our study demonstrated the absence of toxicity in STP2 $\Delta$  strains through OD<sub>600</sub> and spotting assays. This is paradoxical to the results of Willingham *et. al.*, (2003). Besides, we did not see toxicity in the parent strain too. This is also contradictory. Because, other recent studies in yeast (Outeiro *et. al.*, 2003) and other model systems such as mice have demonstrated over expression of  $\alpha$ -synuclein to be toxic (Yamada, 2004). The absence of the toxicity should not be because of the strains; since both Willingham *et. al.*, (2003) and us used the 4741 based STP2 deletions and galactose based  $\alpha$ -synuclein induction systems. However, the vectors used by Willingham *et. al.*, (2003) was p426, where as we used the pYES2 vector, which posses minor differences in the sequence. This might affect the galactose based induction system to such an extent that the optimal amount of  $\alpha$ -synuclein necessary for being toxic in STP2 $\Delta$  background may not be expressed. Optimal amount of misfolded proteins is a crucial factor for toxicity or aggregate formation (Outeiro *et. al.*, 2003). Because, if the optimal amount of  $\alpha$ -synuclein is not present, the protein quality-control system of cells, including ubiquitin ligase (Kitada, 1998), Hsp 70 (Hartl, 2002), and ubiquitin C-terminal hydrolase (Leroy, 1998; Liu, 2002) may cope up with misfolded  $\alpha$ -synuclein (Berke, 2003; Taylor, 2002). To investigate the effect of mutant  $\alpha$ -synuclein in inducing toxicity, we extended the study by over expressing the mutant variants of  $\alpha$ -synuclein--A30P & A53T, and the artificial double mutant A30P/A53T. Similar to the over expression of WT  $\alpha$ -synuclein, the mutant forms are also shown to be lethal (Outeiro *et. al.*, 2003). However, we did not see any difference in the growth rate of the parent and STP2 $\Delta$  strains. This might also be accounted by the difference in the vector system we used for over expressing  $\alpha$ -synuclein.

### STP2 does not appear to influence *in vivo* and *in vitro* $\alpha$ -synuclein aggregation

We next examined the presence of any abnormal phenotypes in parent and STP2 $\Delta$  strains by GFP microscopy. Between the two types, we did not see any difference in the phenotypes (Fig. 4) thereby showing the neutral effect of STP2 deletion. Previous studies have shown that  $\alpha$ -synuclein is a plasma membrane specific protein (Wersinger *et. al.*, 2003). The parent and STP2 $\Delta$  strains over expressing WT  $\alpha$ -synuclein and it mutant form A53T showed halos suggesting the localization of  $\alpha$ -synuclein in the plasma membrane. This indicates that the A53T mutation is neutral to the plasma binding of  $\alpha$ -synuclein. The cytoplasmic localization of  $\alpha$ -synuclein in the plasma membrane for parent and STP2 $\Delta$  strains with the A30P mutant and the double mutant A30P/A53T suggests the necessity of the 30<sup>th</sup> proline for plasma membrane binding. Our results agree with other studies that have conducted similar experiments (Outeiro *et. al.*, 2003; Wang *et. al.*, 2004). Evaluation of any variation in the dominant phenotype at the 48<sup>th</sup> hour did not demonstrate any phenotypic differences. This suggests the absence of the progress of any lethal or unusually phenotypes that are undetectable at the 24<sup>th</sup> hour.

Although the dominant phenotypes were plasma membrane and cytoplasmic localization, we also observed foci in the cytoplasm of parent and STP2 $\Delta$  strains over expressing WT, and A53T. However, the percentage of foci was very low. This further indicates the absence of the optimal amount of  $\alpha$ -synuclein required for being toxic, if foci are the cause of toxicity in the study by Willingham *et. al.*, (2003). Because, simply doubling the amount of  $\alpha$ -synuclein is shown to change the membrane localization of  $\alpha$ -synuclein to cytoplasmic foci (Outeiro *et. al.*, 2003). Quantification of this at the 24<sup>th</sup> and 48<sup>th</sup> hours showed a decrease in the number of foci for parent strain with WT  $\alpha$ -synuclein and an increase for STP2 $\Delta$  with WT  $\alpha$ -synuclein and A53T mutant. An increase in foci was also seen for parent strain with A53T mutant. The question of whether the foci are aggregates of  $\alpha$ -synuclein remains to be answered. However, the absence of toxicity highlights the hypothesis of the 'Protective Function of Aggregates' (Goldberg, 2000). Whether the foci are  $\alpha$ -synuclein aggregates or not, the decrease in foci for parent strain with WT suggests the activation of chaperone systems that removes them (Hartl, 2002). However, a definitive pattern cannot be formulated, since the number of foci is increasing for STP2 $\Delta$  with WT  $\alpha$ -synuclein and its mutant form A53T and also for parent strain with A53T mutant. To make further conclusions, the experiment needs to be repeated. But it suggests the existence of a molecular overlap in the signal transduction pathways of the three types of strains that shown an increase in foci formation at the 48<sup>th</sup> hour.

Although, aggregation is not a prerequisite for  $\alpha$ -synuclein toxicity (Goldberg, 2000), it is possible that the toxicity demonstrated by Willingham *et. al.*, (2003) was due to  $\alpha$ -synuclein aggregation. Besides, *in vitro* studies by Conway *et. al.*, (1998) have shown that  $\alpha$ -synuclein mutations A30P and A53T accelerate the oligomerization of  $\alpha$ -synuclein. Recent studies on yeast have also shown difference in the expression levels of  $\alpha$ -synuclein with A30P mutant having elevated expression over WT and A53T (Outeiro *et. al.*, 2003). We investigated aggregate formation by immunoblotting for  $\alpha$ -synuclein on the cells lysates prepared from parent and STP2 $\Delta$  strains. However, we did not see any difference on the size and on the expression level of  $\alpha$ -synuclein. The absence of aggregates or multimers might also be because of the absence of adequate amount of  $\alpha$ -synuclein necessary for fibrilization due to the induction system we used.

### Relevance of GAPDH

Based on Eckert-Boulet *et. al.*, (2004), we proposed that the enhanced toxicity in STP2 $\Delta$  (Willingham *et. al.*, 2003) was due to GAPDH over expression. However, immunoblotting demonstrates the absence of any difference in the expression level of GAPDH between the parent and STP2 $\Delta$  strains. The absence of the difference might be because of the difference in the strains used. Eckert-Boulet *et. al.*, (2004) used the yeast strain *gap/ura3* M4054, where as we used 4741. Or else it might be because of the high signal-to-noise ratio of the DNA Micro array system used by Eckert-Boulet *et. al.*, (2004) leading to a false positive signal for GAPDH.

Our initial hypothesis was the role of GAPDH in STP2 dependent  $\alpha$ -synuclein toxicity was demonstrated to be invalid. Therefore, we proposed that deletion of GAPDH3 might enhance the growth in both parent and STP2 $\Delta$  strains, if  $\alpha$ -synuclein toxicity is independent of STP2 yet dependent on GAPDH. Similar studies in mice had resulted in the rescue of dopaminergic neurons undergoing apoptosis (Fukuhara *et. al.*, 2001). However, we did not demonstrate any enhanced growth in both parent and GAPDH $\Delta$  strains. This could be explained by the fact that there might not have been any toxicity in the first place because of the absence of optimal amount of  $\alpha$ -synuclein necessary for being toxic. Or



else, GAPDH might not have any link between apoptosis or  $\alpha$ -synuclein in *S. cerevisiae*.

Conclusion

We conclude that the absence of enhanced STP2 dependent  $\alpha$ -synuclein toxicity in our study was due to the difference in the vector used. Our results propose a model where the pYES2 based induction systems expresses  $\alpha$ -synuclein below the optimal amount that is required to bypass the protein quality -controlled systems of the cell

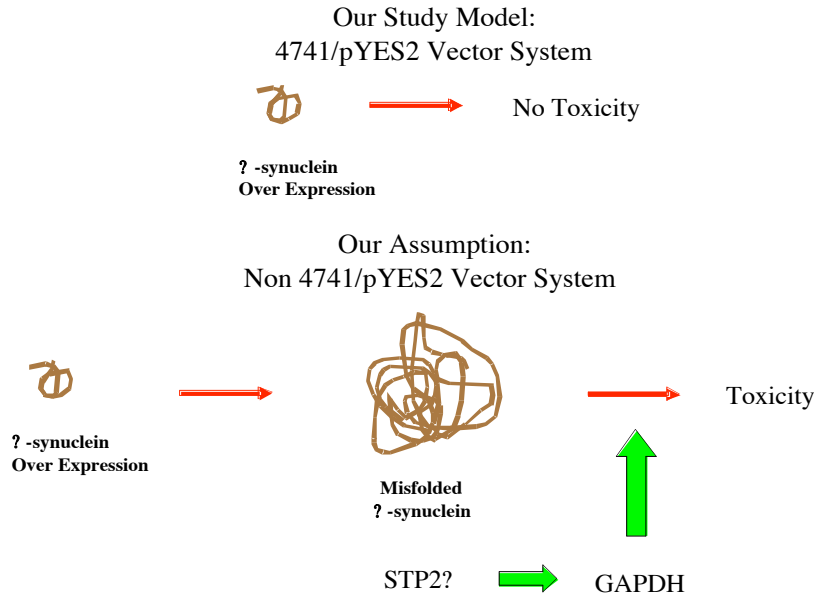


Figure 8. Molecular Model that Links STP2,  $\alpha$ -synuclein and GAPDH

We predict that the absence of STP2 dependent  $\alpha$ -synuclein toxicity as shown by Willingham *et. al.*, (2003) in our study was due to the difference in the vector system used. In our system (4741/pYES2),  $\alpha$ -synuclein expression was not adequate enough to pass the protein quality-control systems in the cell. Also, the GAPDH expression levels did not change in our STP2D strains, which was paradoxical to the observation of Eckert-Boulet *et. al.*, (2004). We assume this difference was because of the difference in the yeast strain used.

(Figure 8). Therefore, to completely verify the role of GAPDH in STP2 dependent  $\alpha$ -synuclein toxicity, the study needs to be repeated with other vectors.

Experimental Procedures

$\alpha$ -Synuclein Constructs

Human wild type and A53T mutant  $\alpha$ -synuclein cDNAs were a gift from Christopher Ross (Johns Hopkins University). A30P and A30P/A53T mutant  $\alpha$ -synuclein were created from wild type and A53T mutant  $\alpha$ -synuclein, respectively, using site-directed mutagenesis (Invitrogen). Wild type and mutant  $\alpha$ -synuclein cDNAs were sub cloned into the pYES2.1/V5-His- TOPO yeast expression vector (Invitrogen). All  $\alpha$ -synuclein forms were also tagged with GFP using a two-step cloning strategy.  $\alpha$ -Synuclein cDNAs were first sub cloned into mammalian expression vectors, pcDNA3.1/C-terminal GFP and pcDNA3.1/N-terminal GFP (Invitrogen).  $\alpha$ -Synuclein-GFP-CT fusion genes were then amplified and subcloned into yeast expression vector, pYES2.1. Chemically competent *E. coli* were transformed with the  $\alpha$ -synuclein and  $\alpha$ -synuclein-GFP expression plasmids and grown on LB-ampicillin media for selection. All constructs were verified by DNA sequencing. The parent pYES2.1 vector (Invitrogen) and GFP in pYES2.1 vector were used as controls.

Expression in Yeast Strains

$\alpha$ -Synuclein expression plasmids were transformed as described (Burke, 2000) into URA-3-deficient, STP2-deficient, and GAPDH3-deficient *S. cerevisiae* strains 4741, 6603, and 4422 respectively. For selection, yeast cells were grown on synthetic-complete media lacking uracil (SC-URA). Presence of  $\alpha$ -synuclein constructs was confirmed by polymerase-chain reaction. The pYES2.1 vector, containing a galactose inducible promoter (GAL1), allowed for regulated  $\alpha$ -synuclein expression. Yeast cells were first grown overnight in SC-URA glucose (2%) media at 30°C. Cells were washed with water and diluted to log-phase in SC-URA galactose (2%) media to induce expression.

Western Analyses

Yeast cells ( $2.5 \times 10^7$  cells/ml) were washed in 50mM Tris (pH 7.5), 10mM  $\text{Na}_2\text{S}_2\text{O}_8$  and solubilized in Electrophoresis Sample Buffer (ESB; Burke, 2000) containing 2% SDS, 80mM Tris (pH 6.8), 10% glycerol, 1.5% DTT, 1mg/ml bromophenol blue, and a cocktail of protease inhibitors and solubilizing agents (1% Triton-X 100, 1mM PMSF, 1mM benzamide, 1mM sodium orthovanadate, 0.7  $\mu\text{g}/\text{ml}$  pepstatin A, 0.5  $\mu\text{g}/\text{ml}$  leupeptin, 10 $\mu\text{g}/\text{ml}$  E64, 2 $\mu\text{g}/\text{ml}$  aprotinin, and 2 $\mu\text{g}/\text{ml}$  chymostatin). Samples (20-30  $\mu\text{l}$ ) were run on pre-cast 10-20% Tris-Glycine SDS gels (Invitrogen), using See Blue (15  $\mu\text{l}$ ) as the molecular standard (Invitrogen). Gels were transferred to PVDF membranes (BioExpress) for 120 minutes at 30V and probed with the antibodies: anti- $\alpha$ -synuclein (Santa Cruz, Biotech), anti- GAPDH antibody (ChemiCon International) and anti-PGK (Molecular Probes); secondary antibody for anti-GAPDH antibody. Antibodies were diluted in blocking buffer as recommended. To detect the antibodies, the following color development solution was used: 100ml Tris Buffer (pH 9.5) containing 15mg/ml BCIP and 30mg/ml of NBT dissolved in 100% and 70% dimethylformamide, respectively (BioRad).

Table 1. Different  $\alpha$ -synuclein Constructs and the Respective Strains Used

Construct	Strain	<i>S. cerevisiae</i> Strain Used
pYES2	Parent	4741
GFP (CT)	Parent	4741
WT- $\alpha$ -synuclein-GFP (CT)	Parent	4741
A30P $\alpha$ -synuclein-GFP (CT)	Parent	4741
A53T $\alpha$ -synuclein-GFP (CT)	Parent	4741
A30P/A53T $\alpha$ -synuclein-GFP (CT)	Parent	4741
pYES2	STP2A	6603
GFP (CT)	STP2A	6603
WT- $\alpha$ -synuclein-GFP (CT)	STP2A	6603
A30P $\alpha$ -synuclein-GFP (CT)	STP2A	6603
A53T $\alpha$ -synuclein-GFP (CT)	STP2A	6603
A30P/A53T $\alpha$ -synuclein-GFP (CT)	STP2A	6603
pYES2	GAPDHA	4422
GFP (CT)	GAPDHA	4422
WT- $\alpha$ -synuclein-GFP (CT)	GAPDHA	4422
A30P $\alpha$ -synuclein-GFP (CT)	GAPDHA	4422
A53T $\alpha$ -synuclein-GFP (CT)	GAPDHA	4422

## Growth Analyses

For OD<sub>600</sub> analysis, yeast cells were grown overnight in SC-URA/Glucose and were washed the next day twice with water and transferred into two sets of cultures (SC-URA/Glucose and SC-URA/Galactose) at early log-phase (5\*10<sup>6</sup> cell/ml). The absorbance readings of yeast cells expressing  $\alpha$ -synuclein at 0hrs, 3hrs, 6hrs, 9hrs, 12hrs, 18hrs, 24hrs, and 36hrs were taken at 600nm using the Hitachi U-200 spectrophotometer. For spotting, yeast cells were grown to mid-log phase in SC-URA raffinose (2%), normalized to equal densities (2\*10<sup>7</sup> cells/ml), serially diluted (five-fold), and spotted on SC-URA glucose (2%) or galactose (2%) plates. Photographs were scanned after 2-3 days of growth.

## GFP Microscopy and Quantification

To analyze cells under fluorescent microscopy, expression was induced for 24 hours as described previously and was diluted to a density of 5\*10<sup>6</sup> cells/ml in 25ml of SC-URA/Galactose media and allowed to grow into log phase. Cells were then washed twice with deionized water and resuspended in 250ul of SC-URA/Glucose media to concentrate them 100-fold. 10 ul of the cells was added to a slide and viewed under the Zeiss Axiovert-100 fluorescent microscope and images were acquired under blue-filter settings using the Metamorph 4.0 imaging software. Quantification was carried out by counting cells in the number of each type of phenotype in six adjacent fields and calculating the ratio of cells displaying certain patterns: general fluorescence (cytoplasmic localization), bright halos (peripheral localization), and foci (possible aggregation) to the total number of cells in the six fields together.

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